Selective Glucocorticoid Receptor Ligands

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Abstract: Glucocorticoids are four-ring steroid compounds that regulate a wide range of physiological systems ranging from embryonic respiratory development, immune function and responses to acute or chronic stress. Glucocorticoids are taken up by many target cells where they bind and activate cytoplasmic glucocorticoid receptors (GRs), which then dimerize, translocate to the nucleus and function as ligand-dependent transcriptional regulators. Synthetic glucocorticoids such as dexamethasone and prednisolone have for decades been the cornerstone for the clinical treatment of inflammatory diseases, such as rheumatoid arthritis and asthma, yet prolonged use have undesirable side-effects such as persistent immune suppression, metabolic imbalance, obesity, diabetes, and osteoporosis. Detailed understanding of the cell signaling mechanisms of GR action has led to the development of novel selective glucocorticoid receptor ligands that appear to offer more efficient treatments for a number of diseases while eliciting fewer side-effects. Additionally, in cell-based and animal model systems a number of compounds such as the methane sulphonamides and a novel compound A-348441 have shown promise as GR antagonists. Other classes of ligands such as the benzopyranoquinolines and the arylpyrazoles have further been shown to selectively influence the transcriptional regulatory properties of GRs on different target gene in various cellular contexts. These selective GR modulators are believed to initiate transcriptional co-regulator recruitment that in turn promotes specific gene responses relevant to the more efficient and specific treatment of inflammatory conditions and metabolic diseases such as type-2 diabetes.

Key Words: Glucocorticoids, glucocorticoid receptor, inflammatory disease, type-2 diabetes, steroid ligand, selective glucocorticoid receptor modulator.

1. INTRODUCTION

Glucocorticoids are members of the family of steroid hormones and are synthesized and secreted by specialized endocrine cells of the adrenal gland. Glucocorticoids are derived from the four ring steroid compound cholesterol and exert specific effects during embryonic development and in the coordinated maintenance of general physiological homeostasis [1, 2]. Glucocorticoids have effects in many adult tissues, helping to regulate processes such as metabolic homeostasis, central nervous system function and immune response modulation. Glucocorticoids are secreted with another important class of steroid, the mineralocorticoids, by the adrenal cortex. Amongst other roles, mineralocorticoids help regulate the balance of systemic electrolytes, such as sodium and potassium [1].

The principal physiological glucocorticoid in humans is cortisol, whereas in rodents it is corticosterone. Cortisol (4-pegnen-11β, 17α, 21-triols-3, 20-dione) is comprised of three 6-carbon hexane rings and a 5-carbon pentane ring backbone, characteristic of all steroid hormones [3]. The presence of the keto-oxygen on carbon 3 and the hydroxylation of carbons 19 and 21 provide the glucocorticoid activity of cortisol [1]. As well as the naturally occurring glucocorticoids, such as cortisol and corticosterone, synthetic glucocorticoids, such as dexamethasone, methylprednisolone and prednisolone, have been developed. These synthetic glucocorticoids have been utilized for decades as therapeutic drugs in various clinical settings. The generic physiological structures of these glucocorticoids, and some synthetic agonists and antagonists are shown in Fig. (**1**). A commonly used synthetic glucocorticoid antagonist, RU486, is also an antagonist of the progesterone receptor and as such is an active ingredient in a widely available oral abortion pill [4]. RU486 has more recently been trialed for treating depression [5].

The primary positive regulator of glucocorticoid synthesis and secretion in the adrenal gland is adrenocorticotropic hormone (ACTH) released from the anterior pituitary gland. ACTH binds the adrenocortical cell ACTH cell surface receptor, activates cell membrane adenyl cyclase and thus stimulates the synthesis of glucocorticoids. Binding of ACTH results in formation of intracellular cyclic-adenosine monophosphate (cAMP) and thus increases the activity of intracellular biosynthetic enzymes, such as the steroidogenic cytochrome P-450$_{scc}$(side chain cleavage), co-activating the catalysis of adrenal steroids [1]. Plasma levels of glucocortcoid hormones are controlled by negative feedback loops. This feedback is mediated by hypothalamic and pituitary glucocorticoid receptor (GR) occupancy that in turn regulates ACTH release, rather than by feedback mediated by target cells. ACTH also enhances the production of adrenal androgens [6]. The release of ACTH from the pituitary is controlled by corticotropin-releasing hormone (CRH) produced and secreted by the hypothalamus [1].

2. GLUCOCORTICOID RECEPTORS

Glucocorticoids effect target cells and tissues *via* the action of two types of specific intracellular cytoplasmic receptor proteins, initially termed the Type I (now termed the mineralocorticoid receptor or MR) and Type II (now termed the glucocorticoid receptor or GR) glucocorticoid receptor.

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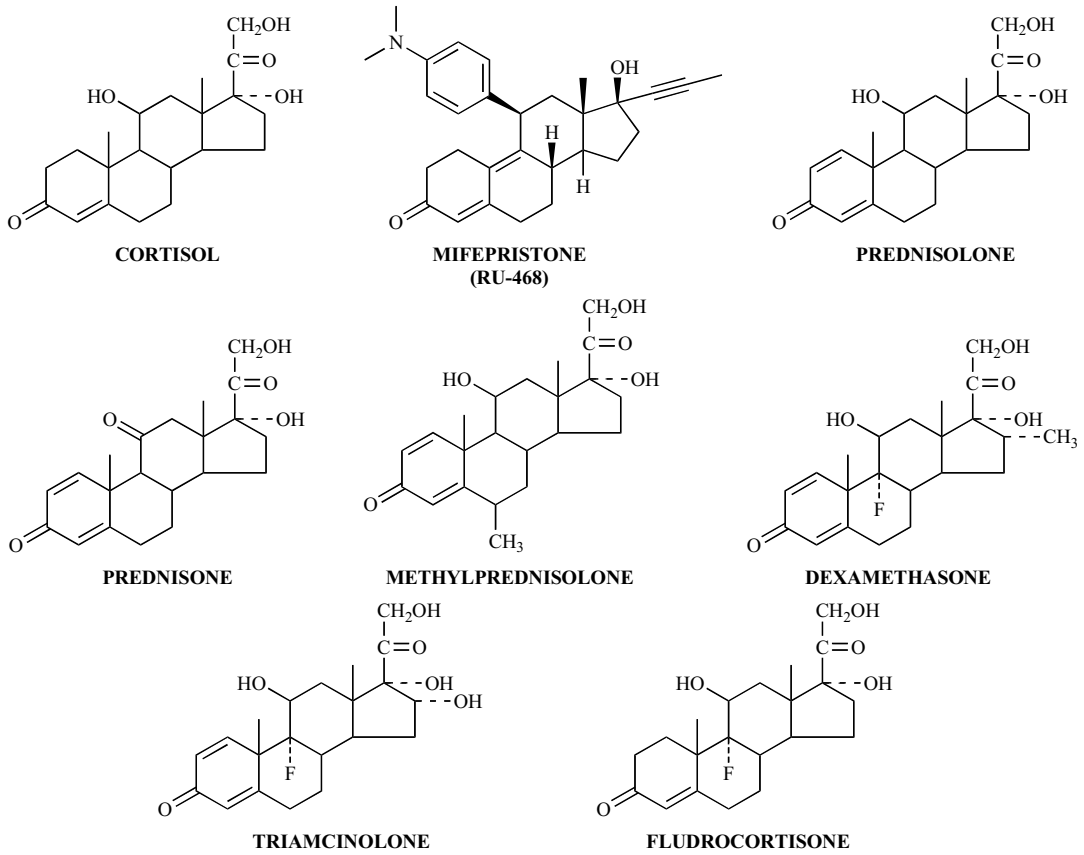

Fig. (1). Chemical structures of common human physiological glucocorticoids (cortisol and cortisone) and synthetic glucocorticoid agonists (prednisilone, prednisone, methylprednisilone, dexamethasone, triamcinolone and fludrocortisone) and the glucocorticoid antagonist RU486, used in clinical treatment of disease.

The GR is expressed to varying degrees in all cell types so far examined and glucocorticoid steroids are its major physiological ligand. The Type I or MR, also binds with high affinity mineralocorticoids such as the physiological adrenal steroid aldosterone. The spatial expression of MR in cells and tissues of the body is more restricted relative to the GR [7]. The MR is present at high levels in tissues containing epithelial cell layers such as the kidney, colon, the parotid gland, salivary glands, ocular tissues, lung, osteoblasts, haematopoetic cells and some regions of the small intestine [8, 9]. A potential role for aldosterone in the pathogenesis of heart failure has recently been suggested from the results of a double blind Randomized Aldactone Evaluation Study (RALES) [10]. In the RALES trial, patients with moderately severe heart failure were given (on top of normal therapy) a low dose of spironolactone, an MR antagonist. After two years, spironolactone produced a 30% reduction in mortality and a 35% reduction in morbidity relative to the control group, the success thus causing the trial to be stopped prematurely. The implied pathology of aldosterone *via* MR in heart failure, either direct or indirect, is poorly understood. The primary role of aldosterone in epithelia such as in the kidney collecting duct or distal convoluted tubule is to facilitate the retention of sodium and water, thereby helping to maintain blood volume and pressure.

GR and MR are members of the steroid receptor subfamily of the nuclear receptor superfamily (shown in Table **1**) and share a common domain structure (Fig. **2**). Both receptors are characterized by a central DNA binding domain (DBD), which functions to target the receptor to specific DNA sequences known as hormone response elements (HREs). The DBD of nuclear receptors is composed of two highly conserved zinc finger motifs. The GR and MR possess almost identical DBDs and can therefore bind identical HREs. The HRE is comprised of two DNA half-sites organised as inverted repeats, mutations of which prevent GR binding [11, 12]. The C-terminal end of steroid hormone receptors possesses the hormone ligand-binding domain (LBD). The N-terminal A/B domain is involved in *trans*-activation of target genes after the receptor complex binds the DNA. In the unliganded state, GR forms an inactive cytoplasmic multi-protein complex with heat shock proteins, such as HSP 90, HSP 70 and HSP 56. These chaperones play a role in assisting a GR folding conformation optimal for hormone binding and preventing the unoccupied receptor from translocating to the nucleus [13, 14]. Upon ligand binding, the chaperones are released and the activated GR or MR dimerizes and translocates to the nucleus where the receptor complex binds to specific DNA hormone response elements in the promoter control regions of target genes and alters the

Table 1. Members of the Human Nuclear Receptor Superfamily of Receptors

Endocrine Receptors (12)	Dietary Lipid Receptors (12)	Orphan Receptors (24)
Glucocorticoid receptor (GR)	Peroxisome proliferator activated-	Retinoid X receptor (RXR) α,β,γ
Progesterone receptor (PR)	receptor (PPAR) α, β, γ, δ	Steroidogenic factor 1 (SF1)
Androgen receptor (AR)	Liver X receptor (LXR α)	Dosage-sensitive sex reversal (DAX-1)
Mineralocorticoid receptor (MR)	Farnesoid X receptor (FXR)	Small heterodimeric partner (SHP)
Estrogen receptor (ER) α, β	Pregnane X receptor (PXR/SXR)	Tailess-related receptor (TLX)
Retinoic acid receptor (RAR) α, β, γ	Const. androstane Receptor (CAR) α, β	Photoreceptor nuclear receptor (PNR)
Thyroid hormone receptor (TR) α, β		NGFI receptors (NGFI-B α, β, γ)
Vitamin D receptor (VDR)		ROR/RZR α, β, γ
		Estrogen-related receptor (ERR α, β, γ)
		Germ cell nuclear factor (GCNF)
		Testis receptor (TR2 α, β)
		Hepatocyte nuclear factor 4 (HNF-4)
		COUP-TF α,β,γ
		Reverse ErbA (RevErb) α, β
		Liver receptor homolog-1 (LRH-1)

rate of gene transcription (see Fig. **3**). Altered expression of these gene products leads to an alteration in cell and eventually tissue function. Specificity of DNA binding is achieved by interaction of the P-box in the first zinc finger of the GR DBD with the palindromic hormone or glucocorticoid response element (GRE) of the target DNA. The GR and MR can also heterodimerize in tissues co-expressing both receptors [14]. This may prove to be an important mechanism as heterodimerization of both corticosteroid receptors expands the potential of these steroid hormones to regulate a larger range of responsive target genes. GR also has several other modes of actions. First, GR can carry out DNA binding-dependent repression of several genes, such as proopiomelanocortin (POMC) and osteocalcin, by binding to specific so-called negative GREs [15]. Second, GR can also modulate the transcriptional activity of certain genes *via* non-DNA binding protein-protein interactions, such as with the AP-1 [16] and NFκB transcription factor complexes [17]. Such regulation involves direct protein-protein interaction between GR and specific subunits of these complexes and is commonly known as "transrepression". Finally, several other mechanisms have been proposed for the possible further cross-talk of GR with other transcription factors such as c-Jun and Stat-5 (extensively reviewed in [18]).

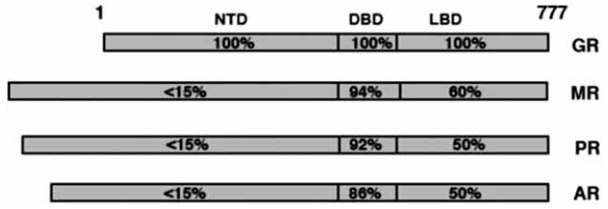

Fig. (2). Comparison of structural domains for members of the steroid receptor subfamily from the nuclear receptor superfamily. NTD; N-terminal domain, DBD, DNA binding domain; LBD, ligand binding domain. GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; AR, androgen receptor. Percentage identity at the amino acid level is related to GR (set at 100%).

3. GLUCOCORTICOIDS AND SYSTEMIC PHYSIOLOGY

In mammals, gluococorticoids regulate a range of physiological systems and in the face of consistent and sometimes sudden changes to the external environment, help to maintain physiological homeostasis. They play an important role during mammalian embryonic development, particularly in the final stages of lung development prior to birth. In the adult glucocorticoids contribute to the regulation of metabolism, strongly repress an activated immune system and modulate brain function during the response to stress [2]. These and other physiological systems where glucocorticoids contribute to regulatory control are depicted in Fig. (**4A**).

3.1. Physiological Role of Glucocorticoids in Mammalian Embryonic Development

The development and growth of the mammalian embryo is a complex and highly organized process involving a combination of intrinsic growth and differentiation factors and concerted actions from circulating factors and hormones. Glucocorticoids are one of a number of circulating hormones, including retinoids, thyroid hormone and other cAMP-mediated factors, that have important roles during the final embryonic stages of lung development. These hormones play key roles in the differentiation and development of terminal respiratory alveoli and in the stimulation of lung surfactant production. Endogenous glucocorticoid levels increase in the fetus rapidly just prior to birth and actively participate in the regulation of biochemical and cytoarchitectural changes in the developing fetal lung, although little is known of the un-

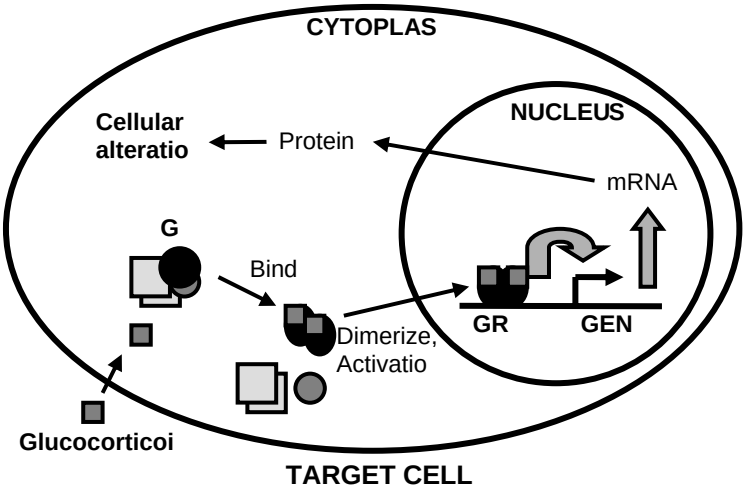

Fig. (3). Signal transduction pathway for the glucocorticoid receptor in target cells. Glucocorticoids enter target cells by diffusion across the cell membrane where they bind the ligand-binding domain of the GR initiating dimerization and translocation to the nucleus. The activated GR dimer binds to specific glucocorticoid response elements (GRE) and modulates the rate of transcription. This produces an elevation of protein level within the cell and eventual physiological change.

derlying molecular and cellular details that underpin these glucocorticoid-mediated effects [19].

To investigate the role of glucocorticoid action *via* GR signaling during embryonic development, the GR gene has been ablated using gene targeting in mice, which caused a number of phenotypic changes [20]. The most striking observation was observed in the lung where GR-null mice at birth displayed severe lung atelectasis with little to no inflation of fetal lung tissue. On a C57Bl6/129sv mouse genetic background, greater than 90% of GR-null mice die at birth from respiratory dysfunction, whereas on a 129sv isogenic mouse genetic background, 100% of GR-null mice die at birth. An identical phenotype of perinatal death and lung dysfunction has been described in a second GR-null mouse constructed by gene-targeted deletion of exon 2 and the proximal promoter of the GR gene [21]. A more recent detailed analysis of the developing lung in the first GR null mouse has shown that glucocorticoid signaling *via* GR is not essential for surfactant production and analysis of alveolar epithelial cell (AEC) -types using electron microscopy and cell-specific markers, revealed a marked reduction in differentiated type-I AECs prior to birth [22]. This indicated that GR signaling is an important epithelial cell differentiation signal that in its absence leads to respiratory dysfunction due to a severe reduction in the ability of the lung to mount and mediate appropriate gas exchange.

An almost identical lung phenotype is seen in corticotrophin-releasing hormone (CRH)-deficient mice (of CRH-deficient mothers) where there is a clear impairment in glucocorticoid production both in the fetus and mother [23]. CRH null mice have delayed induction of surfactant protein-A and surfactant protein-B with reduced pulmonary septal thinning and airway formation. Interestingly, mice with a gene-targeted point mutation in the *GR*, that prevents dimerization and DNA binding, develop normally with no overt lung defect, indicating that the actions of glucocorticoids in the developing lung may be mediated by DNA-binding independent actions [24]. A recent study using GR null mice with deletions of exon 2 and the proximal promoter have found a marked reduction in the growth factor midkine, three days before birth, that may contribute to the immature lung phenotype detected at birth [25]. It is not then surprising that synthetic glucocorticoids (particularly betamethasone and dexamethasone) are widely used antenatally to reduce the severity of the respiratory distress syndrome (RDS) suffered by very pre-term infants and act to accelerate fetal lung maturation and increase lung surfactant production [26]. Antenatal glucocorticoid treatment has had a major benefit in reducing the incidence of neonatal RDS and intraventricular hemorrhage, leading to decreased neonatal mortality. Its use, however, remains controversial, particularly the administration of multiple doses, due to the reported side effects of glucocorticoids on lung and body growth and the postnatal development of the central nervous system (CNS) [27, 28].

3.2. Maintenance of Homeostasis

Glucocorticoids are one of a large number of important components, including the catecholamines, CRH and a large number of cytokines, interpreting and effecting the endocrine response to systemic stress [2, 29]. As mediators of the neural stress response system, glucocorticoids are central in priming systems within, for example, the hippocampus and amygdala, for the fight or flight response and crucial in mediating a return to homeostasis [30, 31]. Short-term responses include promoting and improving memory and increasing acuity. Long-term chronic stress can produce atrophy and damage to the hippocampus, a response mimicked by high glucocorticoid administration to animals.

Although GRs are expressed in most cells of the brain, both GR and MR are co-expressed at the highest levels in limbic neurons that include the hippocampal CA1 and dentate gyrus, as well as nuclei of the amygdala and medial prefrontal cortex [32, 33]. In these areas they have important

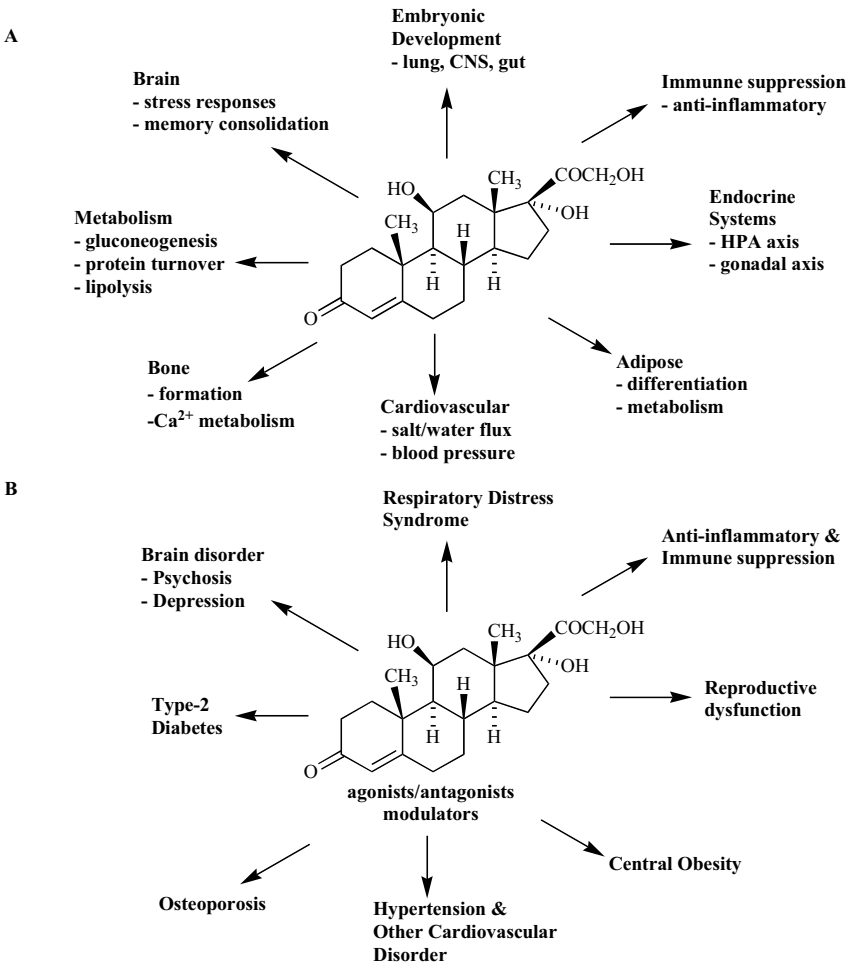

Fig. (4). Panel A: The major physiological functions of glucocorticoids in the humans. CNS, central nervous system; HPA, hypothalamus-pituitary-adrenal. Panel B. The primary diseases and medical conditions that could be treated by tissue-selective glucocorticoid receptor agonists, antagonists, or selective modulators.

roles in the control of emotion and cognition. The hippocampus plays an important role in memory formation and consolidation. In neurons of the hippocampus, MR acts principally as a receptor for glucocorticoids, exerting effects on memory and behavioural and stress responses. Blockade of GR by selective antagonists has shown that GR is important for memory consolidation [34]. These results have been confirmed with gene-targeted GR knockout mice where memory formation was impaired [35]. Recent over-expression of GR in the pre-frontal cortex of mice produced increased anxiety-like behaviour akin to bipolar disorder [36]. Clearly changes in the specific levels of GR in regions of the brain, either pharmacologically or *via* increases or decreases of gene expression will affect stress-related brain function.

Cortisol is released from the adrenal gland in response to a range of stressors that include trauma, infection, intense heat or cold, injection of drugs (norepinephrine, necrotizing substances and other sympathomimetic drugs), surgery and physical restraint [37]. The HPA axis of the neuroendocrine system interprets stress stimuli and the consequences of chronic or acute stress are dependent on the modulated response of the HPA axis [4]. The responsiveness of the HPA axis to stress is dependent on the ability of corticosteroids to regulate CRH and ACTH release [38]. Much evidence suggests that glucocorticoids inhibit HPA activity, not only at the hypothalamus and pituitary, but at higher CNS centers. Lesions of the hippocampus, which contain the highest density of both MR and GR, are associated with failed attenuation of the HPA stress response, circadian rhythm and increased CRH and vasopressin expression [39]. The importance of the GR in negative feedback sensitivity of the HPA axis has been demonstrated in tissue-specific GR knockout mice lacking the GR throughout the CNS [40]. A lack of GR function in the nervous system impaired the regulation of the HPA axis in these mice, resulting in increased plasma glucocorticoid levels that lead to similar clinical symptoms observed in Cushing's syndrome.

3.3. Glucocorticoids and Metabolism

In general, glucocorticoids are catabolic and have significant effects on carbohydrate, protein and lipid metabolism. In the metabolic response to stress or after chronic glucocor-

ticoid administration there is a rapid increase in blood glucose levels. This is promoted in a number of ways. Glucocorticoids alter glucose metabolism by both decreasing glucose utilization in the periphery, and increasing glucose production and availability *via* stimulating hepatic gluconeogenesis [2]. In the liver, glucocorticoids stimulate gluconeogenesis *via* increasing the transcription rates of genes such as phosphoenolpyruvate carboxykinase, tyrosine amino transferase and glucose-6-phosphatase all of which are key enzymes in the gluconeogenic pathway [41]. Glucocorticoids also mobilize amino acids from peripheral tissues such as muscle and adipose, and also reduce the rate of glucose utilisation in cells by decreasing the oxidation of NADH, thereby lowering the rate of glycolysis. Glucocorticoids are also known to reduce protein synthesis and increase catabolism of protein stores in many cells of the body by repressing the formation of RNA and the transport of amino acids in many peripheral tissues such as muscle and lymphoid tissue. Glucocorticoids also stimulate the mobilization of fatty acids from adipose tissue. The dramatic and counter-regulatory effects glucocorticoids have on insulin action have suggested that blockade of glucocorticoid effects *via* antagonizing the GR may be a potential treatment for diabetic hyperglycemia.

3.4. Glucocorticoids and the Immune Response to Challenge

An immune defence against a variety of harmful pathogens is critical to survival in an ever changing biological environment. Mammals have a highly developed and exquisitely ornate immune system able to mount a robust activated response to pathogenic challenge. Activated immune responses however if left to continue unchecked can be detrimental to the host, therefore there are important molecular mechanisms that are activated to suppress such immune responses. Glucocorticoids play key roles inactivating immune responses, particularly aspects driven by the actions of pro-inflammatory cytokines. At moderate to high systemic levels, glucocorticoids have a potent immunosuppressive effect [13, 29]. This is achieved not only by blocking the synthesis of pro-inflammatory cytokines but also by blocking their systemic effects. Glucocorticoids inhibit the synthesis of certain cytokine receptors such as the IL-2 receptor [42]. Also, glucocorticoids can inhibit the cellular effects of certain cytokines such as IL-2 and IL-6, by repressing expression of the transcription factors *Ap-1* and *NF$_\kappa$B*, hence repressing inflammation [43]. Glucocorticoids decrease the number of eosinophils and lymphocytes in the blood and a major effect of cortisol is the inhibition of T cell proliferation [44]. Glucocorticoids also induce apoptosis of immature thymocytes (immature thymic T cells) and some B cells *via* apoptosis [45]. One of the most wide-spread clinical uses of glucocorticoids is for the treatment of rheumatoid arthritis, a discovery and treatment that led to the award of the 1950 Nobel Prize for Physiology and Medicine to Hench, Kendall and Reichstein [46]. Synthetic glucocorticoids are potent inhibitors of inflammation, allergy and endotoxic shock [47]. They inhibit the synthesis of cytokines, such as tumour necrosis factor (TNF) and many cytokines (ie, IL-1 and IL-6) in macrophages, which fuel immune and inflammatory responses, and also interact with lipocortin-1, a member of the annexin family of calcium and phospholipid binding proteins [48].

Studies on the influence of glucocorticoids on the immune system have focused on their role during stress or following pharmacological administration (reviewed in [13]). There are clear differences in potency between natural and synthetic glucocorticoids. For example, the most commonly used glucocorticoid in studies of the immune system, dexamethasone, is a synthetic hormone that exerts a twenty-five times greater suppression of IL-6 or IL-1 levels than a comparable amount of the naturally occurring glucocorticoid, cortisol [49, 50]. Studies that have examined normal physiological concentrations of glucocorticoids demonstrate that glucocorticoids prime the immune system and aid in its function during the early phases of the immune response. These studies have relied upon adrenalectomized animals to examine T cell responses in the absence of adrenal hormones. T cells from adrenalectomized rats were found to respond weakly to concanavalin A, however the replacement of low level glucocorticoids restored T cell responses to that observed in sham operated rats [51]. Similarly, a low level of glucocorticoid administered before endotoxin injection actually increased the subsequent secretion of TNF-α and IL-6 [52]. IL-1, IL-6 and TNF-α are pro-inflammatory cytokines that are released at high levels by multiple cell types during the stress response. These molecules are the most potent stimulators of CRH production and can induce high levels of glucocorticoid production within hours [53, 54]. Such highly elevated stress-induced levels of glucocorticoids cause a profound atrophy of the thymus, and also diminish the size of peripheral immune tissues, yet all other tissues remain intact [2, 55].

At high stress-induced levels, glucocorticoids act to dampen and generically reduce the immune response, including the prevention of a further driving of cytokine activation, the priming of lymphocytes by APCs and a reduction in the responsiveness of already activated cells. Glucocorticoids have a uniform inhibitory effect on the synthesis, release and efficacy of the pro-inflammatory cytokines IL-1, IL-2, IL-6, IL-8, IL-11, IL12, IFN-γ and TNF-α [56]. Glucocorticoids also inhibit the effects of cytokine in target cells, and in the case of IL-2, IL-4, IL-7 and IL-15, do so by acting directly on the JAK-STAT pathway component STAT5 [42]. Separate studies have also reported that the GR can interact with STAT3 and STAT5 to reduce IL-6 signaling [57, 58]. It is currently not known if diurnal levels of GCs influence cytokine signaling during T cell development and homeostasis, or if these effects are confined to the stress response. Glucocorticoids also inhibit antigen processing and the expression of MHC class II by dendritic cells, as well as preventing the migration of dentritic cells by reducing associated adhesion molecule expression [59, 60].

The interaction of GR with two particular transcription factor complexes, Activator protein-1 (AP-1) and NF-κB, mediates most of the anti-inflammatory effects of glucocorticoids and also explains the effects of glucocorticoids on genes that do not contain specific GREs in their promoters. AP-1 is primarily composed of two subunits named c-Fos and c-Jun, and is a pro-inflammatory transcription factor inducible by a number of cytokines [61]. The activated GR can interact with c-Jun, in a protein-protein interaction, termed transrepression, to prevent c-Fos/c-Jun interactions

[62]. Furthermore, the c-Jun/GR heterodimer can still bind to AP-1 response elements in promoter regions to prevent the transcription of AP-1 target genes. Such transrepression is well demonstrated by the example of glucocorticoid-mediated repression of c-Jun expression, whose promoter does not contain any GREs, yet requires intact AP-1 binding sites [63]. A second study showed a similar process upon the POMC promoter, which also lacks a GRE but is inhibited by glucocorticoid treatment. Here it was demonstrated that the GR can directly bind to Nur77, another member of the nuclear receptor superfamily, and subsequently to its response elements, to prevent POMC transcription [64].

A separate mechanism for gene suppression utilizes a composite GRE, which contains a binding site for the GR closely associated with that of a different transcription factor. The CRH promoter contains an AP-1 site closely located to a GRE and binding to this composite GRE by an AP-1/GR complex is a potential mechanism for the suppression of CRH expression and thus the production of glucocorticoids [65]. Glucocorticoids can also inhibit the MAP kinase JNK, by an unknown mechanism, which prevents the phosphorylation and thus activation of c-Fos/c-Jun dimers already present in the cell [66]. The inhibition of cytokine production is mediated mainly through the interaction of the GR with NF-κB [67]. The GR can inhibit NF-κB actions by two mechanisms, the transcription of a family of specific NF-κB inhibitors (IκBs) or inhibition of NF-κB activity by direct protein-protein binding [60, 68, 69]. The exact residues in the GR DNA-binding domain necessary for this direct interaction with NF-κB are not the same as those required to repress AP-1 [70].

The transcription factors NF-κB, CREB and NFAT are essential for T cell activation and, at least in the case of NFAT, positive selection of double-positive thymocytes in the thymus [71]. These factors are dependent on direct interaction with transcriptional co-activators such as CBP/p300 to mediate their effects [72, 73]. These coactivators are also essential for the transcriptional activation of the GR [74], leading to the suggestion that competition for co-activator binding may be a method of GR-mediated repression [75]. Alternatively, recent findings favour a model where GR directly binds to the RelA sub-unit of NF-κB, without preventing its binding to its specific DNA element, but represses the transcription of its target genes by direct protein interactions with the basal transcriptional machinery [76, 77]. However, multiple recent studies involving overexpression of various transcriptional co-activators or mutation of coactivator binding sites in the GR demonstrated no loss of transrepression of AP-1 or NF-κB [78-80]. Additionally, despite being unable to recruit coactivators, antagonist (RU486) bound GR has be shown to maintain the ability to transrepress NF-κB [78]. Thus, despite their role as integrators of GR signaling and that of a variety of other transcription factors, present evidence leans strongly against a role for transcriptional co-activators as limiting factors in transrepression. It is currently not clear if glucocorticoids mediate these effects at their diurnal levels, or only during the stress response.

There have been several separate observations suggesting a role for glucocorticoid signaling in the selection of immature T cells in the thymus. First, high glucocorticoid levels can cause pronounced atrophy of the thymus [81] and second, glucocorticoid signaling could rescue T cell hybridomas from the apoptosis caused by T cell receptor stimulation and vise versa [82]. As the major selection events that occur during thymocyte development are dependent on the level of TCR stimulation received, the fact that glucocorticoids could antagonize signaling from the TCR suggested it was possible that glucocorticoids were involved in T cell development. This was supported by studies that the cloned glucocorticoid-inducible genes from T cells, GILZ and GITR prevented TCR-induced cell death when expressed at high levels in T cells [83]. It was also observed by immuno-histochemistry [84], or RT-PCR [85], that steroid biosynthetic enzymes appeared to be expressed in stromal cells of the thymus. Further studies in the mouse thymus demonstrated using RT-PCR that every enzyme necessary to produce glucocorticoids was present, albeit at much lower levels compared to the adrenal gland [85]. A more recent report has investigated the effect of a rat GR transgene specifically expressed in mouse T cells using the conditional tetracycline-inducible expression system with a human T cell-specific CD2 promoter [86]. They showed in adrenalectomized (to exclude systemic glucocorticoids) transgenic mice, that there is a dramatic increase in thymocyte death by apoptosis after doxycycline-induction of the GR transgene, implicating induction of T cell death from endogenously produced glucocorticoids. This was replicated *in vitro* using a thymic organ culture system and the increased death *in vivo* and *in vitro* could be at least partially blocked by the GR antagonist RU486. These results give compelling evidence for the presence of thymic-produced glucocorticoids that, at least in this transgenic system, are able to trigger significant thymocyte apoptosis.

While it is difficult to imagine that such limiting levels of enzymes produce substantial amounts of glucocorticoids, it was proposed that the thymic stroma may only need to produce glucocorticoids in specific microenvironments where the local concentrations created may be sufficiently high to antagonize TCR signaling. To further investigate a possible role for glucocorticoids in T cell selection, transgenic mouse lines were created that expressed the 3' untranslated region of the rat GR mRNA molecule in an anti-sense orientation under the control of the T cell-specific *Lck* promoter [87]. Thymocytes from these mice possessed a 50% reduction in GR mRNA and displayed defective thymocyte development in the adult that could be detected from as early as day 16 of gestation. Both TCR-β selection (a proliferative stage between the double negative and double positive thymocytes), as well as positive selection appeared to be impaired, leading to a 10-fold reduction in double positive thymocyte number, with very few mature thymocytes or peripheral T cells.

To explain these effects on thymocyte development the mutual antagonism hypothesis was proposed (reviewed in [88]). This hypothesis postulated that TCR signaling in developing thymocytes caused a deletion-inducing signal, which could be antagonized by GR signaling. Only those thymocytes whose TCR signaling was balanced with GR signaling would be positively selected, while less or more TCR signaling than this positive selection threshold would cause glucocorticoid-mediated death by neglect or TCR mediated negative selection, respectively. A prediction of this

hypothesis is that thymocytes that would normally be positively selected, would be deleted if GR-signaling was reduced. This prediction was confirmed in two different models that found increased thymocyte sensitivity to deletion following inhibition of GR signaling. When anti-sense GR mice were crossed to the MRL background, the characteristic autoimmunity of this line was prevented [89]. Additionally, the pigeon cytochrome-c peptide antigen specific T cells normally found in wild-type mice were deleted when crossed to anti-sense GR mice [90].

Despite this strong evidence of a role for glucocorticoids during the positive selection of thymocytes, there was contrasting data reported in a separate line of GR-antisense transgenic mice. Here, the same anti-sense construct was expressed under the control of the neurofilament promoter, which also caused a 50% decrease in GR levels, yet a small increase in double positive thymocytes was observed [91]. A separate group compared these transgenic anti-sense GR mice, and found that both the promoters induced on increased thymocyte number [92]. Separate studies also reported decreased thymocyte sensitivity to deletion when GR signaling was reduced by the GR antagonist RU 486 [93, 94]. Collectively these experiments suggest a role for GR signaling in thymocyte selection and development. However a number of recent experiments with mice lacking GR have shown these conclusions to be most likely incorrect. Studies using GR knockout or mutant mice, have failed to show an influence of glucocorticoid signaling on thymocyte development, selection, or survival [21, 24, 95-98]. The observations of normal thymocyte development has been confirmed in separate studies that have used either a global GR deficient mouse model [21, 95], or a mouse in which the GR has been specifically made deficient in only T cells [97, 98]. In the global GR deficient mouse model, normal negative selection was further confirmed with *in vitro* models of this process involving challenge by staphylococcal enterotoxin B or CD3/CD28 stimulation, and demonstrated that GR signaling is most likely not required for this process. Taken together, these data from GR null mice demonstrate that TCR-based thymocyte selection steps are not reliant on GR signaling. Clearly, this remains an area of ongoing controversy that requires further investigation [99-103].

4. GLUCOCORTICOID RECEPTOR LIGANDS

Recent advances in the cellular mechanisms of GR action, together with the determination of the tertiary structure of the GR ligand binding domain [104], have promoted great interest recently in the development of novel tissue selective ligands that include GR antagonists and differential GR agonists, to selectively modulate glucocorticoid receptor actions to potentially treat specific diseases [105, 106]. This has been driven by the ever present clinical problems associated with chronic treatment of patients with the currently available synthetic glucocorticoids, such as prednisolone and dexamethasone. The myriad of effects glucocorticoids have in many systems in the body makes directed treatment for a specific disease difficult without eliciting major side-effects that include osteoporosis, weight gain, glaucoma and neuropsychiatric symptoms [106]. This has created interest in the development of better glucocorticoid agonists and antagonists, that have no or reduced side effect profiles. A list of currently used glucocorticoid agonists, antagonists and novel compound groups in development are shown in Table **2**. A number of studies have screened large numbers of non-steroidal compounds and used modification chemistries of lead structures to refine novel selective glucocorticoid receptor modulators (SGRMs) [107, 108]. The following section summarises a number of classes of compounds that have been recently published, and outlines their properties, as well as their potential use in treating glucocorticoid-related conditions.

4.1. Early Glucocorticoid Receptor Agonists

Since the availability of synthetic cortisone in the late 1950s, a number of useful synthetic glucocorticoid agonists have been developed for use in clinical practice. These include the synthetic steroids prednisolone, dexamethasone and betamethasone, that have been primarily used for their powerful anti-inflammatory and immune-suppressive effects. All are more potent than cortisol, have longer half-lives, and have differences in anti-inflammatory activity and side-effect profile [1]. They also have less cross-reactivity with the related mineralocorticoid receptor and therefore less salt-retaining and hypertensive side-effects. These agonists can be given orally or by various topical routes (skin, inhaled, rectal). Most synthetic steroids have low affinity for circulating carrier proteins (ie corticosteroid-binding globulin) and circulate as free steroid (up to 30%) or bound to albumin. Chronic administration of synthetic agonists is common in respiratory and arthritic disease and it has been estimated that up to 0.5% of the western population are now prescribed chronic glucocorticoids [1]. Some common synthetic glucocorticoid agonists are:

Prednisolone

A similar chemical structure to cortisol and has a four times more potent anti-inflammatory effect. Prednisolone is widely used for the treatment of rheumatoid arthritis in clinical practise and for a range of other related inflammatory conditions.

Dexamethasone

Addition of a 9α-fluoro group, a 1, 2 saturated bond, and a 16α-methyl group to cortisol produces dexamethasone, a 25 times more potent glucocorticoid than cortisol. It is also widely used as an anti-inflammatory agent and is used in laboratory-based research for the studying the mechanisms of glucocorticoid action, particularly in immune suppression, fetal development and neurological stress-related actions.

Betamethasone

This steroid is similar in structure to dexamethasone but instead has a substituted 16β-methyl group. It is widely used in nasal aerosols for asthma and in the treatment of respiratory distress syndrome in very preterm babies.

4.2. Glucocorticoid Receptor Antagonists

Ligand antagonists of GR are of clinical use for the treatment of hypercortisolemia such as in Cushing's disease and have recently been trialled in the treatment of depression.

RU486

This steroidal antagonist, also called mifepristone, has been available for many years [109] and is currently being used in the treatment of some psychiatric disorders [5]. It does however cross-react with progesterone receptors, preventing widespread use. This has however led to its use as an abortion pill for the termination of early pregnancy by antagonising progesterone receptors in the ovary and uterus.

AL082D06

A non-steroidal compound, AL082D06 was recently developed as a more selective GR antagonist [108]. It is characterized by a tri-aryl methane core, binds to GR with a nanomolar affinity (see Table **2**) and does not cross-react with other related steroid receptors such as MR or PR. AL082D06 was shown to antagonise both glucocorticoid-mediated transcriptional activation and repression. AL082D06 failed to induce formation of GR-GRE complexes or promote binding to DNA, and ligand binding initiated much reduced GR nuclear translocation [108]. AL082D06 exhibited very little agonist activity and therefore is an example of a pure GR antagonist. This compound may be of great clinical use for the treatment of a number of cortisol-related endocrine diseases.

MP-Acid Conjugates

These compounds are cholic acid conjugate derivatives of RU486 (Mifepristone) and have a high affinity for GR [110, 111]. One of these early compounds, A-348441, was shown to be a very good antagonist of GR, having a strong antagonistic effect on glucocorticoid-up-regulated genes and an ability to normalise post-prandial glucose in a rat model of diabetes. A-348441 also reduced hepatic glucose output having no effect on peripheral glucose uptake in a number of animal models [110].

Sulphonamides

Another class of non-steroidal compounds recently developed as a passive GR antagonist have been sulphonamide derivitives [107, 112, 113]. A series of non-steroidal passive N-(3-dibenylamino-2-alkly-phenyl)-methanesulphonamide GR modulators have been described. A number of these compounds have been recently analysed for beneficial effects in liver selective antagonism of the GR and in rodent models of type-2 diabetes [113]. These compounds had a nanomolar affinity for human GR and did not bind significantly to other members of the steroid receptor family. Two compounds effectively lowered plasma glucose, cholesterol and free fatty acid levels, and also reduced weight gain in the *ob*/*ob* mouse model of type-2 diabetes. These compounds did not significantly activate the HPA axis in unstressed mice or have abortive effects on pregnant mice, indicating that these passive GR antagonists may have utility for treatment of type-2 diabetes and other aspects of the metabolic syndrome.

4.3. Glucocorticoid Receptor Modulators

Successful treatment of acute and chronic inflammatory disease is compounded by many unwanted side-effects such as obesity, diabetes, osteoporosis and psychosis. Many of these effects are mediated by the transactivating function of GR on gene targets while most of the anti-inflammatory effects are repressive in mechanism. This prompted a number of research groups to develop GR ligands with dissociated profiles for transactivation and trans-repression, that would lead to a much reduced side-effect profile *in vivo*. Listed below are a number of recently described GR modulators currently under analysis.

ZK 216348

This is a nonsteroidal selective GR agonist that shows significant dissociation between transactivation and trans-

Table 2. Glucocorticoid Ligands Currently in Clinical use or Under Development as SGRMs

Ligand	~GR K_i (nM)	Effect of Ligand	Clinical Disease for Treatment
Dexamethasone	1.2	Agonist	Arthritis, anti-inflammatory
Prednisolone	2.4	Agonist	Arthritis, anti-inflammatory
RU486	0.3	Antagonist	Psychiatric disorders
AL082D06[3]	210	Antagonist	ND
Methanesulphonamides	4.8	Antagonist	Type-2 diabetes
A-348441[7]	0.3	Antagonist	Type-2 diabetes
AL-438[1]	2.5	Modulator	specific anti-inflammatory
ZK216348[2]	20.3	Modulator	Anti-inflammatory
Benzopyranoquinoline	1-6	Modulator	Anti-inflammatory
Phenylpyrazoles[6]	0.7-14	Modulator	ND

SGRMs, selective glucocorticoid receptor modulators; ND, not determined [1] [115]; [2] [114]; [3] [108]; [4] [113]; [5] [117], [6] [120], [7] [110].

repression in cell-based assays and in animals *in vivo* [114]. ZK 216348 had a nanomolar affinity for GR but also bound PR and MR as well. It showed anti-inflammatory activity comparable to prednisolone in a mouse model of systemic and topical inflammation. There was a better side-effect profile with respect to blood glucose increases, activation of liver gluconeogenesis (as measured by tyrosine aminotransferase activity), splenic involution, and to a degree skin atrophy. There was however activation of the HPA axis. This compound is therefore a promising SGRM candidate for treatment of acute inflammatory conditions with an improved therapeutic index and reduced side-effect profile.

AL-438

This compound was derived from a synthetic progestin scaffold compound and finally selected on its superior properties from a series of related compounds [115]. It binds to GR with a very low nanomolar affinity (Table **2**), but also binds MR at relatively high affinity. When tested *in vivo* AL-438 had full anti-inflammatory potency (similar to prednisolone) but had negligible effects on bone metabolism and did not produce hyperglycemia in orally treated rats. Interestingly when given together, AL-438 was able to antagonise the effect of prednisolone in this assay. The mechanism driving these selective effects was shown be differential recruitment of nuclear cofactors with reduced interaction with PGC-1 (an important coactivator for upregulation of hepatic glucose production) and normal interaction with another nuclear GR co-activator GRIP1. AL-438 may represent a selective non-steroidal ligand for specific treatment of inflammatory disease.

Benzopyranoquinolines

These compounds are nonsteroidal GR ligands and were derived from a tetracyclic (benzopyrano[3,4-*f*]quinoline) scaffold designed generally for nuclear hormone receptors [116]. C-10 substitutions conferred GR selectivity and C-5 substitutions conferred modulation of transcriptional activity [117]. Many C-5 substituted compounds showed nanomolar affinity for GR with high selectivity over PR. Comparisons of repression of E-selectin versus activation of a GRE in transfection assays showed that a number of compounds had dissociated activities for transrepression and transactivation [118]. In cell based assays these nonsteroidal modulators had significantly reduced transcriptional activation *via* GREs of aromatase and tyrosine aminotransferase, but maintained almost 100% efficacy with the transcriptional repression of interleukin-6, collagenase and PGE2. These compounds also show promise as selective antiinflammatory drugs but require more testing *in vivo* to fully assess their dose response and effectiveness for the reduction of GR transactivation-mediated side-effects.

Arylpyrazoles

A series of related arylpyrazole compounds have recently been developed and tested in a number of biological cell-based systems for selective GR-mediated effects [119, 120. These compounds have a range of relatively high affinities for GR and some have clear selectively of other related steroid receptors]. Most excitingly though, these compounds when tested across three cell lineages (preadipocyte, preosteoblast and lung epithelium) demonstrated profound differences in the modulation of transcriptional regulatory activity of the GR. These observations require further testing in animal model systems.

5. SUMMARY

There are potential benefits in the development of novel glucocorticoids with better efficacy for the treatment of inflammatory disease and diabetes. Reducing side-effects in respect to enhancing their specificity, would allow more long term systemic use for the treatment of inflammatory conditions such as rheumatoid arthritis. Selective modulators have been developed for other nuclear receptors. The best example is the development of the mixed agonists/antagonists for the estrogen receptor [121]. The selective estrogen receptor modulators tamoxifen and raloxifene have different agonist/antagonist profiles depending on the specific tissue. Such properties have lead to the use of raloxifene as a selective estrogenic drug in the clinic for the treatment of osteoporosis. Similarly, selective thyroid hormone receptor modulators are being developed to treat various thryroid disorders [122], with one of these, termed NH-3, showing promise as a selective thyroid hormone antagonist [123].

Approaches to find novel selective GR ligands have included the screening of very large chemical libraries and the design of specifically modified ligands from the known corticosteroid steroid chemical structure [108, 119]. The isolation of the pure GR antagonist AL082D06 came after the screening of many compounds using a GR-based cotransfection transcriptional assay [108]. Further characterisation showed that this antagonist inhibited glucocorticoid-mediated transcriptional regulation and competed with other steroids for binding to the LBD of GR, where it failed to induce the correct conformational change to allow recruitment of cofactors for agonist activation. Recent determination of the structure of the GR LBD by Bledsoe and co-workers has provided further insight into the basic mechanisms of GR function, ligand selectivity and coactivator recruitment [104]. The human GR LBD was crystallised in a complex with dexamethasone and a TIF2 coactivator peptide (LxxLL). The three dimensional structure was similar to other nuclear receptor LBDs but contained some unique features including a distinct dimerization interface and a unique steroid binding pocket. Similar to PR [124] and unlike ER, the dimerization interface does not involve contact with the LBD helix 10 but involves, *via* β-turns of strands 3 and 4, formation of a central hydrophobic intermolecular β-sheet. This novel dimerization interface is critical for both transactivation and repression functions of the receptor. The GR LBD structure also reveals a distinct ligand binding pocket that contains an additional side-pocket due to the different positioning of helix 6 and 7 [104]. The high sequence homology of MR in these regions suggests that MR may also contain this side-pocket in its LBD. This side-pocket in the GR LBD allows the binding of glucocorticoids over other steroids while its absence in ER, PR and AR explains why these receptors do not. The difference in GR and MR selectivity may be due to differences in hydrogen bond formation perhaps involving residues Q642 of GR that interacts with the 17α-hydroxy group of dexamethasone. The corresponding leucine residue in MR would not promote this interaction and may explain the difference in ligand specificity. Crystallization of the MR LBD

is eagerly awaited. Determination of GR LBD structures with other ligands, both agonist and antagonists will shed important light on the conformational changes that these ligands induce and the effect on coactivator/corepressor recruitment.

A promising selective GR modulator is the non-steroidal compound AL438, that has full anti-inflammatory effects but is a partial agonist [115]. Coactivator recruitment studies showed that AL438 was able to induce an interaction with GRIP-1 with an equal efficacy to prednisolone but recruited the key hepatic coactivator PGC-1 with an efficacy much reduced relative to prednislone. This reduced interaction was also demonstrated in GST-pull down interaction assays and indicates that the structural conformational changes induced by AL438 are different to those of prednisolone, and differentiates interactions between specific coactivators. This also helps to explain the strong anti-inflammatory activity yet a weak hyperglycemic effect seen in animal studies. In line with these results the importance of specific GR surfaces for the activation of particular sets of target genes has recently been analyzed using domain-disrupted GR in ostoesarcoma cells and gene microarrays [125]. Activation of different sets of genes was shown to require different domains or surfaces of the GR further implicating coactivator context-specific transcriptional regulation.

Other avenues for the design of selective ligands need to be considered. These may be extended to interactions with GR surfaces outside the LBD. The coactivator binding surface, that contains a groove for interaction with the coactivator, may be utilized for the design of small molecule inhibitors. Small differences in nuclear receptor coactivator binding surfaces could be used as targets for selective ligands [126]. For example desethylamiodarone, a metabolite of amiodarone, an anti-arrythmic drug, may regulate TR activity by interacting at a site distinct to the TR LBD [127]. The dimerization interface may also be used as a target for inhibiting receptor function. In fact studies in a gene-targeted mouse mutant of GR, where dimerization of GR is blocked, indicates that interruption of GR homodimerization may be effective in separating the activation and repression functions of GR *in vivo* [128].

In summary, further characterization of selective glucocorticoid receptor ligands is required, but there is promise for the development of a number of safer compounds particularly for the treatment of inflammatory diseases. These compounds should have better side-effect profiles and will allow safer therapeutic use for a range of common medical conditions that include type-2 diabetes and inflammatory arthritis.

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